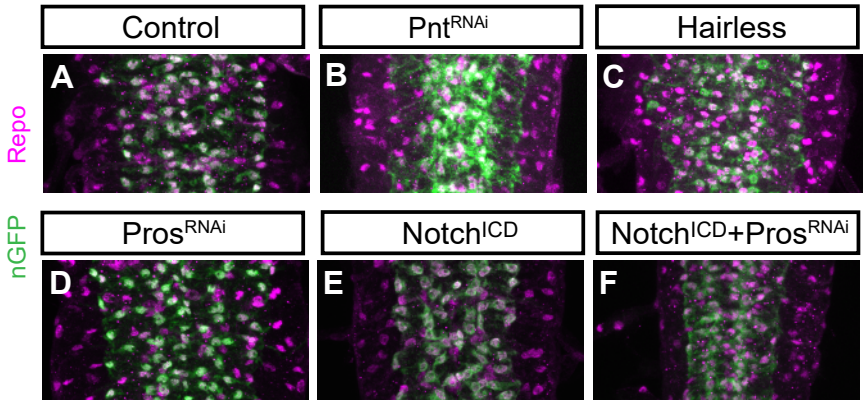

G

Genotypes: *R25H07-Gal4,UAS-nGFP*	GFP+/Repo+ cells per segment (mean ± s.e.m.)	
	L1	L3
Control	18.8 ± 0.1	19.4 ± 0.6
UAS-Pointed RNAi	17.1 ± 0.8*	n.a.(†)
UAS-Hairless	18.2 ± 0.2	19.9 ± 0.6
UAS-Prospero RNAi	18.2 ± 0.3	20.4 ± 0.4
UAS- Notch^{ICD}	18.9 ± 0.2	20.3 ± 0.3
UAS- Prospero RNAi ; UAS- Notch^{ICD}	17.9 ± 0.1	19.8 ± 0.4

Figure S1. Effect of Pointed, Notch signaling or Prospero on glial cell number.

(A-F) L1 VNCs of *R25H07-Gal4,UAS-nGFP* (nGFP; green) immunostained for the glia-specific marker Repo (magenta). All panels show maximum projections of confocal Z-stacks.

(A) Control. (B) Pointed-RNAi. (C) Inhibition of Notch with Hairless. (D) Prospero RNAi. (E) Activation of Notch signaling with Notch^{ICD}. (F) Simultaneous activation of Notch and knockdown of Prospero.

(G) Quantification of GFP+/Repo+ cells per segment (n=5 VNCs for each condition). At L1 stage, only Pointed RNAi induces modest reduction in the number of GFP+/Repo+ cells (ANOVA, $F_{5,24}$=2.766, p=0.0036). *, p<0.05 compared to control with Dunnett's post-hoc test. At L3 stage, Pointed RNAi animals were not available due to lethality (n.a.(†)), and there were no differences among the other groups (ANOVA, $F_{4,20}$=0.8299, p=0.5218).

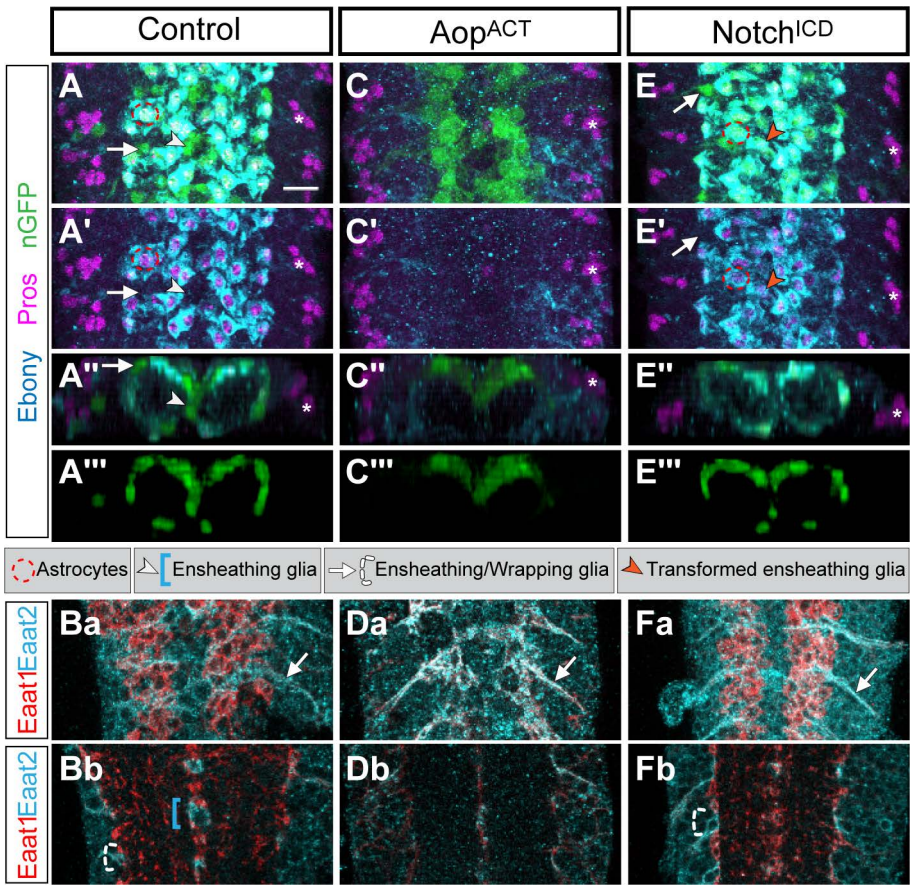

Figure S2. PntP1 inhibition (with Aop^{ACT}) and Notch activation in late-stage embryos.

(A-E''') Co-labeling of LG nuclei in late stage 17 embryos using *R25H07-Gal4,UAS-nGFP* (nGFP, green) together with Prospero (magenta) and Ebony (cyan). Panels (A-A')-(E-E') are horizontal views of maximum projections of confocal Z-stacks, and panels (A''-E'')-(A'''-E''') are the corresponding transverse 3D reconstructions. Asterisks pinpoint neurons. Scale bar, 10 µm. (Ba-Fb) Co-labeling for Eaat1 (red) and Eaat2 (cyan). Single-Z sections at dorsal (Ba-Fa) or medial (Bb-Fb) levels in the neuropil.

(A-Bb) Control. In A-A''', astrocytes express Prospero and Ebony (red stippled circles) but ensheathing glia (arrowheads) and ensheathing/wrapping glia (arrows) do not. Eaat1-positive processes of astrocytes infiltrate the neuropil (Bb). Eaat2 is expressed on ensheathing/wrapping glia (arrow in Ba and white bracket in Bb) and ensheathing glia (blue bracket in Bb).

(C-Db) PntP1 inhibition with *UAS-Aop^{ACT}*. In C-C''', Prospero and Ebony expression are dramatically reduced, and LG positioning is abnormal. Eaat1-positive processes are not seen in the neuropil (Db) and co-expression of Eaat1 and Eaat2 increases on surrounding membranes (Da).

(E-Fb) Notch activation with Notch^{ICD} induces ectopic expression of Prospero and Ebony in ensheathing glia (orange arrowheads in E-E''') but not in ensheathing/wrapping glia (arrows). Eaat2 expression is lost on ensheathing glia membranes. Compare Eaat2 staining near the midline in control (Bb, blue bracket), with its absence near the midline in Fb.

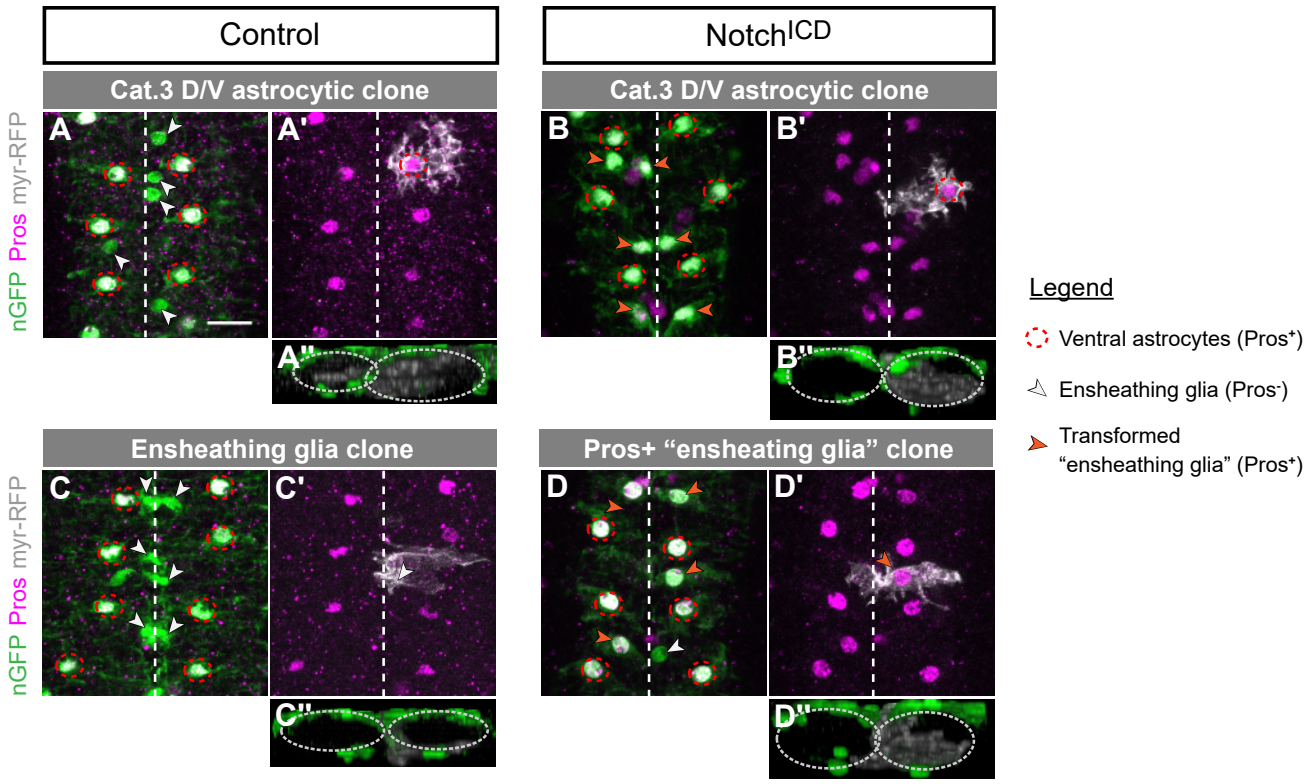

Figure S3. Blown-OUT clones with activated Notch signaling.

R25H07-Gal4 was used to label glial cell nuclei GFP (green), and 2-cell Blown-OUT clones with myr-RFP (white). Prospero (magenta) labels astrocytes. Images are horizontal Z-sections at the most ventral level of the neuropil, except A"-D", which are transverse views at the level of the clones. Dashed vertical lines mark midline. Dotted ovals outline neuropil. Scale bar, 10µm. Legend applies to all panels.

(A-A") Control 2-cell astrocyte clone (Cat.3 D/V) in which both cells express Prospero and infiltrate the neuropil (only the ventral cell is shown in A'). (B-B") Constitutive activation with Notch^{ICD} (N^{ICD}) has no obvious effect: Cat.3 astrocyte clones resemble controls. (C-C") Control 2-cell ensheathing glia clone. Neither cell expresses Prospero, but both envelop the neuropil. (D-D") Constitutive activation of Notch causes ensheathing glia clones (based on cell position) to induce Prospero expression and develop ramified processes that infiltrate the neuropil.